Identification Method of Drought Resistance on Maize Based on qRT-PCR

Xinyue Liu

Hebei Agricultural University

Songtao Liu

Hebei Agricultural University

Tinashe Zenda

Hebei Agricultural University

Yafei Wang

Hebei Agricultural University

Jiao Li

Hebei Agricultural University

Anyi Dong

Hebei Agricultural University

Yatong Yang

Hebei Agricultural University

Huijun Duan (✉ hjduan@hebau.edu.cn)

Hebei Agricultural University <https://orcid.org/0000-0003-0900-3884>

Methodology

Keywords: Zeamays L., Drought, Transcriptome sequencing, Differentially expressed genes, Identification method

Posted Date: October 1st, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-77359/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Identification method of drought resistance on maize based on qRT-PCR

Xinyue Liu^{1,2}, Songtao Liu^{1,2}, Tinashe Zenda^{1,2}, Yafei Wang^{1,2}, Jiao Li^{1,2}, Anyi Dong^{1,2}, Yatong Yang^{1,2}, Huijun Duan^{1,2*}

Abstract

Background: In order to reveal the mechanism of drought resistance of maize, establish the molecular identification system of drought resistance and solve the problem of difficult identification of drought resistance of maize. Taking Nongdan 476, a drought-tolerant maize hybrid cultivar, and Zhongxin 978, a drought-sensitive maize hybrid cultivar, as materials, drought stress and normal watering treatment were carried out at seedling stage, flare stage, tasseling stage and filling stage, and leaf tissue were collected for transcriptomes.

Results: P -value < 0.05 was selected as the screening standard. There were 6281, 17191, 21790 and 15475 differentially expressed genes of each stage, respectively. At the same time, only DnaJ gene was significantly differentially expressed at the four stages. The preliminary results of qRT-PCR showed that the up regulation of DnaJ gene expression was consistent with the results of transcriptome analysis. DnaJ gene expression was further detected by using control samples with specific drought resistance index of yield. The results showed that DnaJ gene expression drought resistance index (DI_E) was highly correlated with yield drought resistance index (DI_P), and the 5-level criteria for DnaJ gene expression drought resistance index (DI_E) were defined by their linear equations.

Conclusion: Drought resistance of maize is a character controlled by multiple genes. There is a significant difference between the expression of DnaJ gene in drought resistant and non drought resistant maize, which has the potential to be used as molecular identification of drought resistance of maize, and can provide a more comprehensive and accurate technical means for drought resistance cultivation and breeding of maize.

Keyword: *Zea mays* L., Drought, Transcriptome sequencing, Differentially expressed genes, Identification method

Background

Drought is the main abiotic factor that restricts the growth and productivity of plants[1,2,3]. Due to climate change, about 70% of the global potential crop yield is lost. With global warming, extreme weather events will become more frequent[4,5]. Therefore, in the coming decades, drought stress is expected to become more

serious[6]. Maize is the world's highest yield food crop. In 2012, the domestic maize production and planting area have exceeded rice and wheat, becoming the largest food crop in China[7]. Maize needs a lot of water in the whole growth period[8,9,10]. The shortage of water resources has a serious impact on the yield and quality of maize[11,12]. It is very urgent to establish the identification method of drought resistance in maize.

The drought resistance performance of maize is restricted by its own genetic effect and environmental factors. Due to the different growth and development period of crops, the influence of biological factors and non biological factors, so far any single drought resistance research has certain limitations, so it is difficult to directly and accurately evaluate the drought resistance of maize. There are many methods to identify the drought resistance of maize[13,14,15], including field direct identification method, artificial simulation environment method, physiological index method and molecular biological identification method. Among them, the field direct identification method is to identify the growth forms of crops in different growth and development periods, but these methods have the disadvantages of long time and environmental factors, especially the large interannual precipitation variation, which makes the research results difficult to repeat. The artificial simulated environment method is to regulate the soil and air in the arid shed, growth box or artificial climate chamber water content is the drought stress environment required by experiments. The method of evaluating the drought resistance of water maize by studying the growth and development of maize, physiological process or the change of yield results is needed. This method needs certain equipment, and the energy consumption is relatively large. The created drought stress environment is different from the field production under natural conditions, which results in the experiment results and the field production. There are some differences in the results of direct identification, physiological index method is mainly used to identify the drought resistance of plants, such as leaf water related indicators, plasma membrane permeability and enzyme activity. However, these physical and chemical indicators will change due to the different growth environment and growth period, and are also prone to errors due to the reagents used and human operation. Molecular biological identification method is based on modern molecular biology, the saturated molecular genetic map was constructed by using related molecular markers, and the drought resistance genes of maize were located. The drought resistance varieties were selected by using molecular markers. Drought resistance is a quantitative character controlled by multiple genes[16]. In recent years, many drought resistance QTLs were found, but the polymorphism frequency of the markers is very low, and a single QTL has little effect on the phenotypic difference, and the difficulty in evaluating epistasis effect. Further

research is needed to use molecular marker assisted selection[17]. Therefore, it is urgent to establish new technologies and find new markers to solve these problems.

In recent years, with the rapid development of omics, transcriptomics has been applied in the field of crop drought resistance research , which provides a development direction for the discovery and screening of molecular markers for drought resistance identification of maize and improving the level of drought resistance identification.

Results

Sample Total RNA Agarose Gel Electrophoresis Detection

Total RNA of 36 samples was isolated from non-stressed and stressed at seedling stage, flare stage, tasseling stage and filling stage of the two maize hybrid cultivar(Nongdan476 and Zhongxin978) and prepared for Agarose gel electrophoresis (Fig.1). It can be seen in the figure that the total RNA electrophoresis bands of 36 samples are clear and bright, and the RNA integrity is good, which is suitable for transcriptome sequencing.

Fig.1 RNA agarose gel electrophoresis of 36 samples of maize leaves

RNA-Sequencing (RNA Seq) Analysis

Total RNA isolated from non-stressed and stressed was used for the RNA-Seq transcriptome analysis. After filtering the sequencing results, a total of 2.03B clean data was generated from 36 samples. For the 36 samples, the bases with the quality value of clean data $\geq Q30$ were higher than 95.95%, which proved the reliability of the sequencing results (Table 1). The clean reads of each sample were mapped to the maize reference genome sequence(B73 RefGen_v3), the results showed that the mapping rates ranged from 84.69% to 93.23% (Table 1).

Table 1 Summary of RNA sequencing results for the 36 samples

Sample	Clean reads	Clean bases	%>Q30	Mapped reads (%)	Multiple map reads (%)	Uniq map reads (%)
HNC1	47610098	7121781293	96.33	43143631 (90.62%)	1771015 (3.72%)	41372616 (86.9%)
HNC2	54166538	8115186558	96.56	50258591 (92.79%)	1921638 (3.55%)	48336953 (89.24%)
HNC3	44085504	6589805593	96.5	38332401 (86.95%)	1959422 (4.44%)	36372979 (82.51%)
HNC4	55009492	8222898730	96.42	51002794 (92.72%)	3181675 (5.78%)	47821119 (86.93%)
HNC5	65161690	9742131458	96.33	58919049 (90.42%)	6515496 (10.0%)	52403553 (80.42%)
HNC6	56622130	8471865241	96.34	52397865 (92.54%)	5421799 (9.58%)	46976066 (82.96%)
HNC7	54963524	8235124779	96.44	50467490 (91.82%)	1963843 (3.57%)	48503647 (88.25%)
HNC8	58848272	8814370506	96.75	54571986 (92.73%)	2208504 (3.75%)	52363482 (88.98%)
HNC9	58068856	8695086186	96.62	53792336 (92.64%)	2018045 (3.48%)	51774291 (89.16%)
HND1	57188372	8564502124	96.62	53180180 (92.99%)	2092360 (3.66%)	51087820 (89.33%)
HND2	59870224	8967789116	96.48	55618716 (92.9%)	2198668 (3.67%)	53420048 (89.23%)
HND3	54580992	8178310681	96.56	49427404 (90.56%)	1986808 (3.64%)	47440596 (86.92%)
HND4	63872458	9551164073	96.54	59337947 (92.9%)	3538578 (5.54%)	55799369 (87.36%)
HND5	57285292	8566007257	95.95	52878662 (92.31%)	3575624 (6.24%)	49303038 (86.07%)
HND6	65497138	9795814869	96.27	60517588 (92.4%)	4793112 (7.32%)	55724476 (85.08%)
HND7	54424602	8153331563	96.63	50537680 (92.86%)	1971162 (3.62%)	48566518 (89.24%)
HND8	59322012	8881605467	96.26	53685546 (90.5%)	2265570 (3.82%)	51419976 (86.68%)
HND9	55801650	8362214149	96.45	51513215 (92.31%)	2168883 (3.89%)	49344332 (88.43%)
ZXC1	56506150	8460982813	96.65	51191380 (90.59%)	1764307 (3.12%)	49427073 (87.47%)
ZXC2	51767644	7756619879	96.63	46450490 (89.73%)	1695231 (3.27%)	44755259 (86.45%)
ZXC3	55162792	8257374897	96.4	50381514 (91.33%)	1963351 (3.56%)	48418163 (87.77%)
ZXC4	69453720	10401048519	96.64	64131601 (92.34%)	4071321 (5.86%)	60060280 (86.48%)
ZXC5	68680646	10281443114	96.48	63822410 (92.93%)	5917064 (8.62%)	57905346 (84.31%)
ZXC6	52541662	7856055799	96.27	48652899 (92.6%)	4214726 (8.02%)	44438173 (84.58%)
ZXC7	50734246	7604425994	96.11	45834069 (90.34%)	2453387 (4.84%)	43380682 (85.51%)
ZXC8	50287034	7535531498	96.31	45665143 (90.81%)	1802850 (3.59%)	43862293 (87.22%)

ZXC9	58917786	8828916182	96.49	53716986(91.17%)	2024127(3.44%)	51692859(87.74%)
ZXD1	55592308	8325795684	96.42	50819133(91.41%)	1979305(3.56%)	48839828(87.85%)
ZXD2	53330596	7988769663	96.49	48023453(90.05%)	1977139(3.71%)	46046314(86.34%)
ZXD3	56881498	8517462308	96.44	51900338(91.24%)	2199950(3.87%)	49700388(87.38%)
ZXD4	61111776	9135032695	96.08	56972442(93.23%)	5532463(9.05%)	51439979(84.17%)
ZXD5	56526168	8462589056	96.43	52511777(92.9%)	4476596(7.92%)	48035181(84.98%)
ZXD6	62732652	9391090136	96.43	57802530(92.14%)	4117858(6.56%)	53684672(85.58%)
ZXD7	47741364	7135040802	96.6	42755846(89.56%)	2130029(4.46%)	40625817(85.1%)
ZXD8	45132964	6753066841	96.01	38223779(84.69%)	3702006(8.2%)	34521773(76.49%)
ZXD9	50628404	7584663563	96.31	44608453(88.11%)	3552265(7.02%)	41056188(81.09%)

Differential Gene Expression Analysis

In order to determine the response of materials to drought, the transcriptome of the same cultivar in the same stage of drought and control treatment were analyzed for differential expression, and in order to explore the genetic differential expression genes of two extreme cultivars, the differential expression genes of two hybrid cultivars under the same water condition were analyzed. Therefore, four sets of differentially expressed genes can be obtained in each treatment stage, namely seedling stage: TC_TD, SC_SD, TC_SC, SD_TD; flare stage: TC1_vs_TD1, SC1_vs_SD1, TC1_vs_SC1 and SD1_vs_TD1; tasseling stage: TC2vs_TD2, SC2_vs_SD2, TC2_vs_SC2 and SD2_vs_TD2; filling stage: TC3_vs_TD3, SC3_vs_SD3, TC3_vs_SC3 and SD3_vs_TD3. With $|\log_2(\text{fold change})| > 1$, $P\text{-value} < 0.05$ as the screening criteria, 6281, 17191, 21790 and 15475 differential genes were expressed at seedling stage, flare stage, tasseling stage and filling stage, respectively (Fig. 2).

Fig. 2 Statistics of differentially expressed genes in different periods

Screening of Marker Genes

When maize is under drought stress, there are up-regulated and down-regulated genes in different development stages and different strains (drought tolerance or sensitivity). Under the same moisture conditions, 4331, 6580, 8180 and 7477 differential genes were identified at seedling stage (TC_SC), flare stage (TC1_vs_SC1), tasseling stage (TC2_vs_SC2) and filling stage (TC3_vs_SC3) before drought treatment, respectively. After the drought treatment, 5398 differential genes were identified at seedling stage (SD_TD), 6282 differential genes were identified at flare stage (SD1_vs_TD1), 10091 differential genes were identified at tasseling stage (SD2_vs_TD2), and 7442 differential genes were identified at filling stage (SD3_vs_TD3). 129, 666, 2417, and 375 differential genes were identified in drought-resistant cultivar (Henong 476) at seedling stage (TC_TD), flare stage (TC1_vs_TD1), tasseling stage (TC2_vs_TD2), and filling stage (TC3_vs_TD3) before and after drought stress treatment. 754, 3663, 1102, and 181 differential genes were identified in the sensitive cultivar (Zhongxin978) at seedling stage (SC_SD), flare stage (SC1_vs_SD1), tasseling stage (SC2_vs_SD2), and filling stage (SC3_vs_SD3) before and after drought stress treatment (Fig. 3). In response to drought stress, some gene sets play a more important role. As shown in the figure, I, II, III and IV represent the differentially expressed genes of resistant cultivar-Henong 476 before and after drought treatment and the differentially expressed genes in response to drought stress in the two hybrid cultivars. Through analysis, we found that a differential gene *zm00001d02666* was identified as a marker gene for co-expression during the four stages of drought treatment, which can be used to identify the drought resistance of Maize (Fig. 4). Moreover, heat maps were drawn for the expression of this differential gene in each group at four different

stages. It could be found that the expression level of drought-resistant cultivar was higher than that of sensitive cultivar after drought stress (Fig. 5).

Fig.3 Venn diagram analysis of the differentially expressed genes at different stages of drought stress, (A-D) were seedling stage, flare stage, tasseling stage and filling stage respectively. The differentially expressed genes of drought-resistant maize hybrid cultivar (I-IV) in response to drought stress were also expressed after drought stress.

Fig.4 Screening the common differentially expressed genes of I-IV part.

Fig.5 Clustering analysis show the expression amount of the marker genes in different groups at different stages. Red represents high expression and green represents low expression.

Verification of Expression Difference of DnaJ Marker Gene

The expression of DnaJ gene at seedling stage, flare stage, tasseling stage and filling stage was verified by using drought-resistant cultivar Nongdan 476 and drought-tolerant cultivar Zhongxin 978 (Fig. 6). It can be seen from Fig. 6 that the expression level of qRT-PCR of drought resistant cultivar Nongdan 476 was high at four stages, while that of non-drought resistant cultivar Zhongxin 978 was low at four stages. It is suggested that RNA-Seq of DnaJ gene is highly consistent with qRT-PCR.

Fig.6 Differential expression and qRT-PCR of DnaJ marker gene in different stages of Maize Development

Expression of DnaJ Marker genes DC_E and DI_E

The DnaJ gene expression levels of 12 maize cultivars under different treatments, the drought resistance coefficient and drought resistance index of the DnaJ gene expression levels are shown in table 2. Those with strong drought resistance are Zhengdan 1002 and Jidan 50; those with medium drought resistance are Xianyu 335, Nonghua 101, Shandan 609, Zhengdan 958, Weike 702, Denghai 605, and Longdan 10; weak drought resistance are Jixiang 1, Shandan 618, Liaodan 588.

Table 2 Drought resistance index of reference sample expression

No.	Cultivar	Expression		DC_E	DI_E	Drought resistance
		Control treatments	Drought stress			
1	Xianyu 335	27.74	27.58	0.99	1.05	MR
2	Nonghua 101	23.07	23.64	1.02	0.93	MR
3	Jixiang 1	27.35	24.85	0.91	0.87	S
4	Zhengdan 1002	25.73	29.08	1.13	1.26	R

5	Shandan 609	27.64	27.51	1.00	1.05	MR
6	Zhengdan 958	26.43	25.49	0.96	0.94	MR
7	Jidan 50	24.82	27.17	1.09	1.14	R
8	Shandan 618	26.32	24.41	0.93	0.87	S
9	Weike 702	24.48	24.87	1.02	0.97	MR
10	Liaodan 588	27.35	24.44	0.89	0.84	S
11	Denghai 605	27.01	26.87	0.99	1.03	MR
12	Longdan 10	25.73	27.08	1.05	1.09	MR

Note: Refer to 2.7 expression quantity drought resistance index division standard for drought resistance

Correlation and Regression Analysis Between Expression of DI_E and Drought Resistance Index DI_P

Microsoft Excel 2013 was used to establish a linear equation between expression DI_E and yield drought resistance index DI_P , as shown in Fig.7. According to the correlation coefficient $r = 0.92$ in Fig.7, the Significant correlation is reached ($P < 0.01$).

Fig.7 Regression and correlation of expression DI_E and drought resistance index DI_P

Through the regression equation $y=0.9982x-0.0998$, substitute the standard value of 5 grades of yield drought resistance index into the formula, and obtain the corresponding standard value of 5 grades of expression quantity drought resistance index, as shown in Table 3. According to table 3, the criteria of drought resistance index of expression quantity are as follows: ≥ 1.30 is extremely strong (HR); 1.11-1.29 is strong (R); 0.91-1.10 is medium (MR); 0.71-0.90 is weak (s); and ≤ 0.70 is extremely weak (HS)

Table 3 Corresponding table of yield drought resistance index and expression drought resistance index of different levels

Drought resistance index	Divide the defined values of the 5 categories of criteria			
Yield drought resistance index x	1.2	1	0.8	0.6
Expression quantity drought resistance index y	1.3	1.1	0.9	0.7

DnaJ Gene for Drought Resistance Identification of Maize Hybrids

See Table 4 for the results of drought resistance identification of maize hybrids by DnaJ gene. It can be seen from table 4 that the expression drought resistance index of DnaJ gene can obviously divide the drought resistance of 40 cultivars into 5 categories, among which the cultivars with extremely strong drought resistance are Xunle 969, Xianyu 7037, Jinlai 376, Wannuo 6 and ND367, the cultivars with strong drought resistance are Denghai 682, Wannuo 161, Bencheng 11, Jiyuan 178 and Xun608, and there are 19 cultivars with medium drought resistance, such as Yufeng 310, Huaiyu 18, Longhua 598 and Chenghua 598, Chengyu 38 and Jike 7118, the cultivars with weak drought resistance are Yufeng 98, Shiyu 1504, Yefeng 703, Tongyu 568, Fuyu 369 and so on. the varieties with weak drought resistance are Dayu 906 and Yijin 229.

Table 4 Identification of drought resistance of marker genes

No.	Cultivar	DI _E	Drought resistance	No.	Cultivar	DI _E	Drought resistance
1	Xunle 969	1.47	HR	21	Fuyu 178	0.96	MR
2	Xianyu 7037	1.40	HR	22	Yudan 9953	0.96	MR
3	Jinlai 376	1.39	HR	23	Xiandai 716	0.94	MR
4	Wannuo 6	1.37	HR	24	Yuanyu 369	0.93	MR
5	ND367	1.31	HR	25	Xingyu 0952	0.93	MR
6	Denghai 682	1.17	R	26	Yufeng 621	0.92	MR
7	Wannuo 161	1.15	R	27	Yufeng 506	0.92	MR
8	Bencheng 11	1.14	R	28	Lidan 20	0.91	MR
9	Jiyuan 178	1.13	R	29	Liyu 112	0.91	MR
10	Xun 608	1.11	R	30	C6361	0.90	S
11	Yufeng 310	1.10	MR	31	Yufeng 98	0.90	S
12	Huaiyu 18	1.02	MR	32	Shiyu 1504	0.90	S
13	Longhua 598	1.02	MR	33	Yefeng 703	0.89	S
14	Chengyu 38	1.01	MR	34	Tongyu 568	0.89	S
15	Jike 7118	0.99	MR	35	Fuyu 369	0.88	S

16	Haiping 605	0.98	MR	36	Ying 986	0.86	S
17	X1501	0.98	MR	37	Jiyu 33	0.86	S
18	Xianyu 1758	0.97	MR	38	Heyu 15	0.85	S
19	Nongdan 145	0.97	MR	39	Dayu 906	0.78	HS
20	Jingkenuo 768	0.97	MR	40	Yijin 229	0.72	HS

Discussion

In this study, in order to explore the response of Maize to drought, we analyzed the transcriptome differential expression of drought stress and control treatment at seedling stage, flare stage, tasseling stage and filling stage. The results showed that the differential expression genes in seedling stage, flare stage, tasseling stage and filling stage were 6281, 17191, 21790, and 15475, respectively. Furthermore, drought resistance is a very complex quantitative trait controlled by multiple genes. The DnaJ gene (zm00001d02666) with significant difference in expression at four stages was screened through linkage analysis. The gene was preliminarily verified in drought resistant and non drought resistant maize by qRT-PCR. The result was up-regulated in drought resistant cultivars and significantly different from that of sensitive materials. This result was consistent with the result of transcriptome analysis, which further suggested that DnaJ gene could be used as drought resistant material. The potential of the identified molecular marker. In addition, this study collected maize cultivars with different drought resistance that have been identified, and detected the expression level of DnaJ by qRT-PCR technology. The results showed that the expression level of DnaJ was significantly different in different drought resistant maize cultivars, and the expression level was positively correlated with the drought resistance index ID of different maize cultivars, and the difference was statistically significant, so it could be used as a marker for drought resistance identification of maize.

DnaJ protein is a kind of protein in Hsp40 family. Its N-terminal contains a conserved J domain of about 70 amino acids, also known as J protein[18]. DnaJ protein can promote the ATPase activity of HSP70, and it is the chaperone of HSP70[18,19]. In the adverse environment, it can complete the correct folding of protein, maintain the stability of peptide chain, and prevent cell damage caused by environmental stress[20]. Some studies have shown that DnaJ protein plays an important role in the life activities of plants to cope with environmental stress[21]. Therefore, it provides a theoretical basis for the selection of DnaJ gene as a marker for drought resistance identification of maize.

Conclusions

The marker DnaJ gene has many advantages in identifying drought resistance. The method is simple. It can identify the drought resistance of Maize by qRT-PCR alone,

and does not need to combine with other characters and indexes. Accurate identification. From the results of this experiment, we can see that the expression level of DnaJ gene has a significant positive correlation with the drought resistance index ID_p of different maize cultivars, which is consistent with the identification results of the field direct identification method widely used at present. It can be used to identify maize hybrids, such as Nongdan 476, Zhongxin 978 and maize inbred lines, such as 8112, zong31 and Mo17. Flexible identification period. It can be identified at seedling stage, flare stage, tasseling stage and filling stage. The qRT-PCR method based on DnaJ gene will provide an advanced technology for the identification of maize drought resistance.

Materials and Methods

Transcriptome Sequencing Materials

Two maize cultivars with contrasting drought sensitivity (tolerant Nongdan 476 and sensitive Zhongxin 978) were used in this experiment. Seeds of the two maize hybrid cultivars were provided by the North China Key Laboratory for Crop Germplasm Resources of Education Ministry (Hebei Agricultural University, China). The experiment was conducted on May 2018 in a drought-resistant shed at Qing Yuan, Baoding, Hebei province, China (N 38°79', E115°56'). The area of the experiment plot was 24m², 60cm row spacing and 30cm plant spacing. Seeds of Nongdan 476 and Zhongxin 978 were sowed by double-grain hole with 6cm depth on the plots fertilized with compound fertilizer 512 kg/hm². In this experiment, the normal watering control group and water stress treatment group were set up for the two experimental materials at seedling stage, flare stage, tasseling stage and filling stage, respectively.

Reference material

Twelve maize cultivars such as Xianyu 335, Nonghua 101, Jixiang 1 were selected as control samples. The drought resistance index and drought resistance are shown in Table 5. For twelve maize materials, a normal watering control group and a water stress treatment group were respectively set. The leaves at the seedling stage are used as experimental materials, RNA is extracted, qRT-PCR analysis were carried out , and the drought resistance index of gene expression was calculated using marker genes to verify the drought resistance of maize.

Table 5 Drought resistance index of corn cultivars

No.	Cultivar	Production				Drought resistance
		Control treatments(kg)	Drought stress(kg)	DC _p	DI _p	

1	Xianyu 335	817.6	718.8	0.88	0.98	MR
2	Nonghua 101	812.3	675.8	0.83	0.88	MR
3	Jixiang 1	791.8	590.6	0.75	0.69	S
4	Zhengdan 1002	791.8	748.2	0.94	1.26	R
5	Shandan 609	791.4	649.4	0.82	1.05	MR
6	Zhengdan 958	778	656.3	0.84	0.94	MR
7	Jidan 50	738.3	699.6	0.95	1.14	R
8	Shandan 618	735.9	557.9	0.76	0.87	S
9	Weike 702	731.6	641.6	0.88	0.97	MR
10	Liaodan 588	729.5	550.8	0.76	0.84	S
11	Denghai 605	716.6	654.2	0.91	1.03	MR
12	Longdan 10	671.3	608.1	0.91	1.09	MR

Note: DCp production drought resistance coefficient (Drought Coefficient, DCp); DIP production drought resistance index (Drought Resistance Index, DIP)

Verification materials

Forty identification materials were selected for stress treatment at seedling stage, of which forty were hybrids promoted in production. Maize seedlings grow under normal conditions until the three leaves are fully unfolded. Then, forty identification materials were subjected to drought stress treatment for 7 days. Half of the plants grew under sufficient water conditions (control group), the remaining plants were subjected to drought stress, and the soil moisture content did not exceed 50% (treatment group). After 7 days of treatment, the leaves treated with drought at the seedling stage were taken as experimental materials. RNA was extracted, and fluorescence quantification was performed. The drought resistance of maize was determined by marker gene.

Measurement of Soil Relative Moisture Content and Sampling

The relative moisture content of maize was measured by measuring the relative moisture content of the soil in the two experimental fields of normal irrigation and water stress. The relative moisture content of the soil in the normal irrigation control group and the moisture stress treatment group was 70-80% and 15-20% respectively. The relative soil moisture content (RSWC) of one meter underground was monitored by soil moisture meter (Zhejiang Top Cloud-Agri Technology CO. Ltd., Zhejiang; China). In the water stress treatment group, the maize plants were treated with drought at the seedling stage, flare stage and the first 10 days before flowering stage, and the plants were treated with drought at the filling stage from pollination. After the soil moisture content was lower than 20% and lasted for 7 days, the top leaves were collected from the control and drought stress treated plants, frozen immediately with

liquid nitrogen, stored in a refrigerator at -80 °C, and analyzed for transcriptome. Each treatment was replicated three times.

RNA Extraction, cDNA Library Construction and Transcriptome Sequencing

Total RNA of the leaf samples was isolated from non-stressed and stressed leaves of the two maize hybrid cultivars using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocols. The RNA was purified by the treatment of RNeasy column (QIAGEN, Pudong, Shanghai, China) to remove genomic DNA. The concentration of RNA was detected by NanoDrop 1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). and the quality of extracted RNA was detected by 1% agarose gel electrophoresis. According to Illumina standard cDNA library , a kit was constructed to construct a chain specific library (on an Illumina Hiseq Xten platform, San Diego, CA, USA), which was sequenced by Novogene Bioinformatics Technology Co. Ltd. (Beijing, China).

Processing, Mapping of Sequencing Reads and Gene Expression Quantification

Raw data (raw reads) generated by the Illumina HiSeq 2000 system contains low-quality sequences with splices. In order to ensure the quality of information analysis, We need to get clean data. In this step, clean data (clean reads) were obtained by removing the sequence with N-base (n indicates that the base information can not be determined), removing the connector sequence in reads, removing the low-quality base ($Q < 20$), and removing the base whose tail quality value is less than 20 using the sliding window method (the window size is 5 bp), so as to obtain clean data. These high quality reads were used all the subsequent analyses. All these clean reads were then mapped to the maize reference genome sequence (B73 RefGen_v3), Tophat 2.0.12 software was used to compare the filtered data[22]. Reads that were compared to known transcriptome and partial reads that were compared were further analyzed and annotated. For functional annotation, the quality reads were used for BLAST(basic local alignment search tool) alignment and annotation against non-redundant protein sequence database (Nr) (<https://www.ncbi.nlm.nih.gov/>), Swiss-port (a manually annotated and reviewed protein sequence database) (<https://web.expasy.org/docs/swiss-prot>); Clusters of Orthologous Groups (COG) (<https://www.ncbi.nlm.nih.gov/COG/>) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) (<http://www.genome.jp/kegg>)[23]. Gene expression levels were calculated and standardized gene expression levels were expressed as RPKM (reads per kilobase of transcript per million mapped reads)[24].

Differentially Expresses Genes (DEGs) Library Construction and Differential Analysis

The DESeq R package (1.10.1)[25] was used to analyze the differential expression of genes[26]. In order to get the genes with significant difference, the screening condition was $P\text{-value} < 0.05$ (P was the adjusted p value < 0.05), and the multiple of difference $|\log_2FC| > 1$. According to the RPKM value of the differential genes, and P -value of each contrast corrected for multiplicity using the Benjamini and Hochberg method[27]. The heat map of their expression quantity in each sample was drawn.

Quantitative real time-PCR (qRT-PCR) Analysis

In order to verify the expression level of the gene *DNAJ* gene detected by Illumina RNA-seq. In this experiment, C1000 (CFX96 Real-time System) Thermal Cycler (Bio-Rad) was used for quantitative real-time PCR (qRT-PCR). Using 1 μg of RNA as a template, perform reverse transcription to 20 μl according to the instructions of the HiFiscript cDNA Synthesis Kit (CWBIO, Beijing, China), and perform qRT-PCR using the reverse transcribed cDNA as a template. In this experiment, Primer Premier 5 Designer (Premier Biosoft International, Palo Alto, CA, USA) was used to design specific primers for differentially expressed genes of the *DnaJ* gene, and the results of the transcriptome were verified. The maize gene *GAPDH* (accession no.X07156) with stable gene expression was selected as an internal reference gene. The qTR-PCR reaction system includes: 2 μl of template cDNA, 0.5 μl of forward primer (50 pmol), 0.5 μl of reverse primer (50 pmol), and 10 μl of SYBR Green mix (TOYOBO, Japan) in a total reaction volume of 20 μl . Each sample had three technical replicates.. The relative mRNA abundance was calculated according to the $2^{-\Delta\Delta CT}$ method[28].

Statistical Data Processing and Analysis Methods

The measured data are collected and processed uniformly by Microsoft Excel 2013. Expression Drought Resistance Coefficient (DC_E) formula: $DC_E = \text{water stress expression amount} \div \text{control expression amount}$; Expression Drought Resistance Index (DI_E) formula: $DI_E = (DC_E \times \text{water stress expression amount}) \div \text{average water stress expression amount of all maize cultivars}$.

According to the 5-level classification standard of yield drought resistance index, ≥ 1.20 is extremely strong (HR); 1.01-1.19 is strong (R); 0.81-1.00 is medium (MR); 0.60-0.80 is weak (s); and ≤ 0.60 is extremely weak (HS) to determine the 5-level classification standard of expression drought resistance index.

Supplementary information

Fig.1 RNA agarose gel electrophoresis of 36 samples of maize leaves

Fig.2 Statistics of differentially expressed genes in different periods

Fig.3 Venn diagram analysis of the differentially expressed genes at different stages of drought stress, (A-D) were seedling stage, flare stage, tasseling stage and filling

stage respectively. The differentially expressed genes of drought-resistant maize hybrid cultivar (I-IV) in response to drought stress were also expressed after drought stress.

Fig.4 Screening the common differentially expressed genes of I-IV part.

Fig.5 Clustering analysis show the expression amount of the marker genes in different groups at different stages. Red represents high expression and green represents low expression.

Fig.6 Differential expression and qRT-PCR of DnaJ marker gene in different stages of Maize Development

Fig.7 Regression and correlation of expression DI_E and drought resistance index DI_P

Abbreviations

RNA seq: RNA Sequencing; DC_E : Expression Drought Resistance Coefficient; DC_P : Production Drought Resistance Coefficient; DI_E : Expression Drought Resistance Index; DI_P : Production Drought Resistance Index; BLAST:Basic Local Alignment Search Tool.

Acknowledgements

The authors of this research would like to thank the North China Key Laboratory for Crop Germplasm Resources of Education Ministry (Hebei Agricultural University, China) for providing transcriptome sequencing materials.

Authors' contributions

XL and HD conceived and designed the experiments. XL performed the bioinformatics analysis and wrote the paper. SL、TZ and HD helped to improve the paper. SL、YW、JL、AD、YY performed the investigations and collected data to improve the experiments. All authors read and approved the final manuscript.

Funding

This research was supported by the National Key Research and Development Project of China (Selection and Efficient Combination Model of Wheat and Maize Water Saving, High Yield and High Quality Varieties) (Grant No. 2017YFD0300901)

Availability of data and materials

Not applicable.

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors agreed to publish this manuscript.

Competing interests

The authors declare that they have no competing interests.

Author details

¹ Department of Crop Genetics and Breeding, College of Agronomy, Hebei Agricultural University, Baoding, China. ² North China Key Laboratory for Crop Germplasm Resources of the Education Ministry, College of Agricultural, Hebei Agricultural University, 071000 Baoding, China.

References

1. Ghatak A, Chaturvedi P, Weckwerth W. Cereal crop proteomics: Systemic analysis of crop drought stress responses towards marker-assisted selection breeding. *Front Plant Sci.* 2017;8:757.
2. Miao ZY, Han ZX, Zhang T, Chen SY, Ma C. A systems approach to spatio-temporal understanding of the drought stress response in maize. *Sci Rep.* 2017; 7:1–14.
3. Trinh T, Ishida K, Kavvas ML, Ercan A, Carr K. Assessment of 21st century drought conditions at Shasta Dam based on dynamically projected water supply conditions by a regional climate model coupled with a physically-based hydrology model. *Sci Total Environ.* 2017;586:197-205.
4. Feller U, Vaseva II. Extreme climatic events: Impacts of drought and high temperature on physiological processes in agronomically important plants. *Front Environ Sci.* 2014;2:39.
5. I.P.C.C. Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change [Core Writing Team, RK Pachauri and LA Meyer (eds.)]. IPCC. 2014. Geneva, Switzerland, 151 pp.
6. Andjelkovic V. Introductory chapter: climate changes and abiotic stress in plants. IntechOpen. 2018, pp 4.
7. Qiu HG, Zhang SH, Yang J, Jing Y. Development of China's Maize Industry, Challenges in the Future and Policy Suggestions. *J Agr Sci Tech-IRAN.* 2013;15:20-24.
8. Min HW, Chen CX, Wei SW, Shang XL, Sun MY, Xia R, Liu XG, Hao DY, Chen HB, Xie Q. Identification of drought tolerant mechanisms in maize seedlings based on transcriptome analysis of recombination inbred lines. *Front Plant Sci.* 2016;7:1080.
9. Zheng J, Fu JJ, Gou MY, Huai JL, Liu YJ, Jian M, Huang QS, Guo XY, Dong ZG, Wang HZ, Wang GY. Genome-wide transcriptome analysis of two maize inbred lines under drought stress. *Plant Mol Biol.* 2010;72:407–421.
10. Yin XG, Olesen JE, Wang M, Kersebaum KC, Chen H, Baby S, Öztürk I, Chen F. Adapting maize production to drought in the northeast farming region of China. *Eur J Agron.* 2016;77:47–58.
11. Maiti RK, Maiti LE, Sonia M, Maiti AM, Maiti M, Maiti H. Genotypic variability in maize (*Zea mays* L.) for resistance to drought and salinity at the seedling stage. *J Plant Physiol.* 1996;148:741–744.

- 12.Li Z, Xu WJ, Xue BD, Cao P. Discuss on evaluating method to drought-resistance of maize in seedling stage. *J Maize Sci.* 2004;12:73–75.
- 13.Bruce WB, Edmeades GO, Barker TC. Molecular and physiological approaches to maize improvement for drought tolerance. *J Exp Bot.* 2002;53:13-25.
- 14.Grzesiak MT, Grzesiak S, Skoczowski A. Changes of leaf water potential and gas exchange during and after drought in triticale and maize genotypes differing in drought tolerance. *Photosynthetica.* 2006;44:561-568.
- 15.Betran FJ, Beck D, Banziger M, Edmeades GO. Genetic analysis of inbred and hybrid grain yield under stress and nonstress environments in tropical maize. *Crop Sci.* 2003;43:807-817.
- 16.Kebede H, Subudhi PK, Rosenow DT, Nguyen HT. Quantitative trait loci influencing drought tolerance in grain sorghum (*Sorghum bicolor* L. Moench). *Theor Appl Genet.* 2001;103:266-276.
- 17.Tuberosa R, Salvi S, Sanguineti MC, Landi P, Maccaferri M, Conti S. Mapping QTLs regulating morpho-physiological traits and yield: case studies, shortcomings and perspectives in drought-stressed maize. *Ann Bot.* 2002;89:941-963.
- 18.Georgopoulos CP, Lundquist-Heil A, Yochem J, Feiss M. Identification of the *E.coli* DnaJ gene product. *Mol Gen Genet.* 1980;178:583-588.
- 19.Hartl FU, Martin J. Molecular chaperones in cellular protein folding. *Nat Rev.* 1996;381:571-579.
- 20.Wang W, Vinocur B, Shoseyov O, Altman A. Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. *Trends Plant Sci.* 2004;9:244-252.
- 21.Cyr DM, Langer T, Douglas MG. Dna J-like proteins: molecular chaperones and specific regulators of Hsp70. *Trends Biochem Sci.* 1994;19:176-181.
- 22.Kim D, Pertea G, Trapnell C, Pimentel H, Kelley R, Salzberg SL. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biol.* 2013;14: R36.
- 23.Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 1997;25:389-3402.
- 24.Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B. Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nat Meth.* 2008;5:621-628.
- 25.Kim D, Langmead B, Salzberg SL. HISAT: A fast spliced aligner with low memory requirements. *Nat Methods.* 2015;12:357-360.
- 26.Anders S, Huber W. Differential expression analysis for sequence count data. *Genome Biol.* 2010;106:10-11.
- 27.Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Statist Soc B.* 1995;57:289–300.
- 28.Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods.* 2001;25:402-408.

Figures

Figure 1

RNA agarose gel electrophoresis of 36 samples of maize leaves

Figure 2

Statistics of differentially expressed genes in different periods

Figure 3

Venn diagram analysis of the differentially expressed genes at different stages of drought stress, (A-D) were seedling stage, flare stage, tasseling stage and filling stage respectively. The differentially expressed genes of drought-resistant maize hybrid cultivar (I-IV) in response to drought stress were also expressed after drought stress.

Figure 4

Screening the common differentially expressed genes of I-IV part.

Figure 5

Clustering analysis show the expression amount of the marker genes in different groups at different stages. Red represents high expression and green represents low expression.

Figure 6

Differential expression and qRT-PCR of DnaJ marker gene in different stages of Maize Development

Figure 7

Regression and correlation of expression DIE and drought resistance index DIP